INCREASE IN CIRCULATING PRODUCTS OF LIPID PEROXIDATION (F_2-ISOPROSTANES) IN SMOKERS

Smoking as a Cause of Oxidative Damage

JASON D. MORROW, M.D., BALZ FREI, PH.D., ATKINSON W. LONGMIRE, M.D., J. MICHAEL GAZIANO, M.D., SEAN M. LYNCH, PH.D., YU SHYR, PH.D., WILLIAM E. STRAUSS, M.D., JOHN A. OATES, M.D., AND L. JACKSON ROBERTS, II, M.D.

Abstract *Background.* It has been hypothesized that the pathogenesis of diseases induced by cigarette smoking involves oxidative damage by free radicals. However, definitive evidence that smoking causes the oxidative modification of target molecules in vivo is lacking. We conducted a study to determine whether the production of F_2-isoprostanes, which are novel products of lipid peroxidation, is enhanced in persons who smoke.

Methods. We measured the levels of free F_2-isoprostanes in plasma, the levels of F_2-isoprostanes esterified to plasma lipids, and the urinary excretion of metabolites of F_2-isoprostanes in 10 smokers and 10 nonsmokers matched for age and sex. The short-term effects of smoking (three cigarettes smoked over 30 minutes) and the effects of two weeks of abstinence from smoking on levels of F_2-isoprostanes in the circulation were also determined in the smokers.

Results. Plasma levels of free and esterified F_2-isoprostanes were significantly higher in the smokers (mean ±SD, 242±147 and 574±217 pmol per liter, respectively) than in the nonsmokers (103±19 and 345±65 pmol per liter; $P = 0.02$ for free F_2-isoprostanes and $P = 0.03$ for esterified F_2-isoprostanes). Smoking had no short-term effects on the circulating levels of F_2-isoprostanes. However, the levels of free and esterified F_2-isoprostanes fell significantly after two weeks of abstinence from smoking (250±156 and 624±214 pmol per liter, respectively, before the cessation of smoking, as compared with 156±67 and 469±108 pmol per liter after two weeks' cessation; $P = 0.03$ for free F_2-isoprostanes and $P = 0.02$ for esterified F_2-isoprostanes).

Conclusions. The increased levels of F_2-isoprostanes in the circulation of persons who smoke support the hypothesis that smoking can cause the oxidative modification of important biologic molecules in vivo. (N Engl J Med 1995;332:1198-203.)

CIGARETTE smoking is a serious health problem worldwide. Smoking has been strongly implicated as a risk factor for chronic obstructive pulmonary disease, cancer, and atherosclerosis.[1-3] Because cigarette smoke is known to contain a large number of oxidants,[4] it has been hypothesized that many of the adverse effects of smoking may result from oxidative damage to critical biologic substances. Such damage could result both from oxidants present in cigarette smoke and from the activation of phagocytic cells that generate reactive oxygen species.[4,5]

Oxidative inactivation of antiproteases may be involved in the development of chronic obstructive pulmonary disease, and oxidative modification of DNA can lead to the development of cancer.[4,6,7] It has been shown that oxidatively modified low-density lipoprotein (LDL), but not native LDL, is recognized by scavenger receptors and taken up by macrophages, a process considered pivotal in the development of foam cells in atherosclerotic lesions.[8] Thus, oxidation of LDL by cigarette smoke may contribute to the causative link between cigarette smoking and atherogenesis. Although previously there was controversy about whether direct exposure of LDL to cigarette smoke in vitro results in oxidative modification,[9,10] we recently demonstrated the formation of lipid hydroperoxides after the exposure of plasma to the gas phase of cigarette smoke.[11] Other evidence suggesting that smokers are subjected to oxidative stress includes the findings that they have lower levels of the antioxidant ascorbic acid (vitamin C) than nonsmokers and that smokers' risk of coronary artery disease correlates inversely with their intake of the antioxidants vitamin E and beta carotene.[12-15]

In spite of the circumstantial data from in vitro studies and measurements of antioxidant levels in smokers suggesting that cigarette smoke may cause oxidative injury, whether this process occurs in vivo has been controversial.[10,16-18] The conflicting evidence can largely be attributed to the fact that most methods previously available to assess oxidative stress in humans have been inaccurate and unreliable.[19]

Recently, we discovered a series of bioactive prostaglandin F_2–like compounds (termed F_2-isoprostanes) that are produced independently of the cyclooxygenase enzyme in humans by the peroxidation of arachidonic acid, catalyzed by free radicals (Fig. 1).[20] F_2-isoprostanes are initially formed in situ on phospholipids and are subsequently released preformed.[21] Levels of F_2-isoprostanes in normal human biologic fluids exceed those of cyclooxygenase-derived prostanoids by approximately one to two orders of magnitude. In addition, levels of F_2-isoprostanes — both free in the circu-

From the Departments of Medicine and Pharmacology (J.D.M., A.W.L., J.A.O., L.J.R.) and the Division of Biostatistics, Department of Preventive Medicine (Y.S.), Vanderbilt University, Nashville; the Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston (B.F., S.M.L.); the Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston (J.M.G.); and the Section of Cardiology, Brockton–West Roxbury Veterans Affairs Medical Center and Harvard Medical School, Boston (W.E.S.). Address reprint requests to Dr. Morrow at the Departments of Pharmacology and Medicine, 506 MRB, Vanderbilt University, Nashville, TN 37232-6602.

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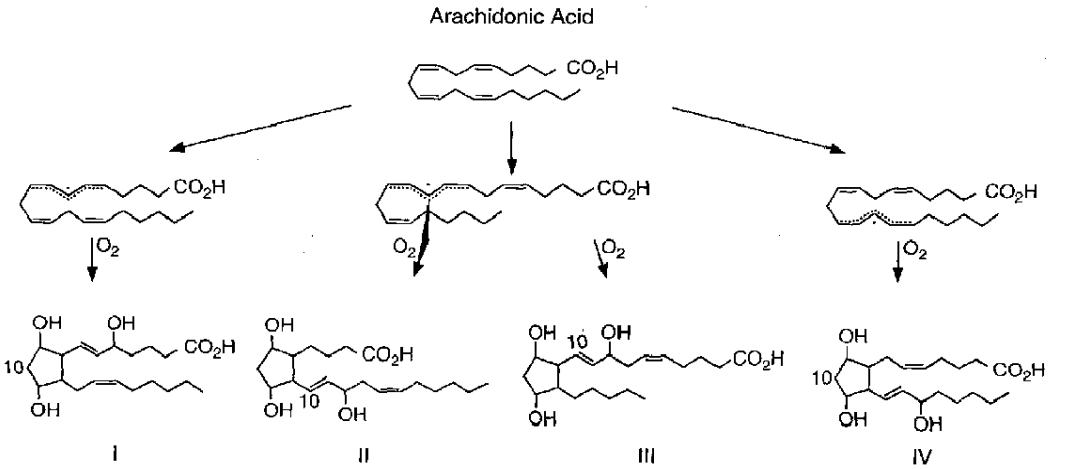

Figure 1. Mechanism of Formation and Structures of the F_2-Isoprostanes.

The F_2-isoprostanes are derived from arachidonic acid (top), which undergoes peroxidation catalyzed by free radicals to yield arachidonyl radical intermediates, which are then transformed to a series of prostaglandin F_2–like compounds composed of four regioisomers (I through IV). Each regioisomer consists of eight racemic diastereomers. The dots outside the molecules indicate unpaired electrons. The lines of dots inside the molecules represent the delocalization of the electrical charge over several carbon atoms. Stereochemistry is not shown.

lation and esterified to tissue phospholipids — increase dramatically in animal models of oxidant injury.[21,22] The most widely used test of lipid peroxidation is the measurement of malondialdehyde with the thiobarbituric acid–reacting substances assay.[19] We recently demonstrated that the F_2-isoprostane level is far superior to measurements of thiobarbituric acid–reacting substances as an index of lipid peroxidation in vivo.[23] Thus, the discovery of F_2-isoprostanes is considered an important advance in our ability to detect oxidant injury.

We undertook this study to assess the hypothesis that cigarette smoking can cause oxidative modification of important biologic molecules in humans by determining whether the production of F_2-isoprostanes is increased in persons who smoke.

Methods

Measurements of F_2-Isoprostanes

Blood was drawn into Vacutainer tubes containing EDTA and immediately centrifuged to separate the plasma. An aliquot of plasma was then removed and stored at −70°C for measurement of lipid-soluble antioxidants. For ascorbic acid and uric acid measurements, the plasma was extracted with metaphosphoric acid before storage at −70°C. The remainder of the plasma was immediately processed for the measurement of F_2-isoprostanes. Levels of free F_2-isoprostanes in plasma and of F_2-isoprostanes esterified to plasma lipids were measured by stable-isotope-dilution mass-spectrometric assay.[24] The precision of the assay for free F_2-isoprostanes is ±6 percent, and the accuracy is 96 percent. The precision of the assay for esterified F_2-isoprostanes in plasma is ±8 percent. Data are expressed in picomoles per liter. We recently identified tetranor dicarboxylic acid urinary metabolites of F_2-isoprostanes that contain one keto group and one double bond with a molecular weight of 328.[25] These metabolites were measured by a mass-spectrometric method, previously described,[25] that has a precision of ±12 percent. Data are expressed in picomoles per millimole of creatinine.

Measurements of Antioxidants in Plasma

Plasma concentrations of ascorbic acid (vitamin C), uric acid, alpha- and gamma-tocopherol (vitamin E), alpha and beta carotene, cryptoxanthin, lycopene, lutein and zeaxanthin, and retinol were measured by high-performance liquid chromatography.[26,27] Data are expressed in micromoles per liter for water-soluble antioxidants and retinol and in micromoles per millimole of total lipids (the sum of cholesterol and triglycerides) for lipid-soluble compounds.

Measurements of Urinary Nicotine and Cotinine

Concentrations of nicotine and its metabolite, cotinine, in urine were quantified by high-performance liquid chromatography; the results are expressed in micromoles per liter.[28]

Clinical Protocols

Two separate clinical studies were performed. Both were approved by the human studies committees of the medical centers involved, and informed consent was obtained from all subjects. Initially, we carried out a pilot study in which we recruited 24 subjects (16 who smoked one to two packs of cigarettes per day and 8 nonsmokers). Twelve of the subjects were apparently healthy; six of the smokers had angiographically or clinically documented coronary artery disease; and six subjects (two nonsmokers and four smokers) had hypertension. Patients with these conditions were receiving medical therapy. No subjects took vitamin supplements. Blood was collected on a single occasion after an overnight fast for measurement of F_2-isoprostanes.

After the completion of the pilot study, we carried out a second, more detailed, validation study for which we recruited 10 smokers (who smoked more than 1.5 packs of cigarettes per day) and 10 nonsmokers matched with the smokers for age and sex. The nonsmokers were not exposed to passive smoke at work or at home. Five men and five women made up each of the study groups. All the subjects included in the study had a normal physical examination, electrocardiogram, and blood-chemistry tests (complete blood count and the 18

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tests of the Sequential Multiple Analyzer 18 system) and had no apparent underlying disease. None were taking medications or vitamin supplements.

The study protocol was as follows. On day 1, the smokers were studied at 8 a.m. and were not allowed to smoke or eat before the first blood sample was obtained. After blood was obtained for measurements of F_2-isoprostanes and circulating antioxidants, the subjects smoked three cigarettes during a 30-minute period, after which blood was again obtained for analysis of F_2-isoprostanes. The same procedure was repeated on day 3 of the study. The average of the two measurements of F_2-isoprostanes in blood obtained on days 1 and 3 is reported here. The median variance in the levels of F_2-isoprostanes measured on separate days did not exceed 12 percent. A 24-hour urine specimen was collected on day 1 for measurement of F_2-isoprostane metabolites; urine was also collected on day 1 for measurement of nicotine and cotinine. For nonsmokers, blood was obtained for measurement of F_2-isoprostanes on days 1 and 3 of the study and for measurement of antioxidants on day 1. Urine was collected on day 1 for measurement of F_2-isoprostane metabolites.

The subjects who smoked were then instructed to cease smoking for a period of two weeks. No nicotine-replacement therapy (such as nicotine patches) was used. Two of the 10 smokers were unable to quit smoking. After the cessation of smoking, blood was again obtained on two separate days for the measurement of F_2-isoprostanes. Urine samples were collected at the end of the two-week period of abstinence from smoking for measurement of nicotine and cotinine to monitor compliance.

Statistical Analysis

In the pilot study, data were analyzed with the Wilcoxon rank-sum test. In the second study, smokers and nonsmokers were matched in terms of age and sex. Data were therefore analyzed using the Wilcoxon signed-rank test. Tests of the Spearman (nonparametric) correlation were used to assess the association of F_2-isoprostane levels with other measurements. All data are expressed as means ±SD. Differences were considered statistically significant if the P value was 0.05 or lower.

Results

Pilot Study

Initially, we compared levels of F_2-isoprostanes in 16 smokers and 8 age-matched nonsmokers. All but one of the subjects were male. The smokers ranged in age from 43 to 72 years (mean, 55.2±8.3). The nonsmokers ranged in age from 47 to 71 years (mean, 54.4±8.7). The smokers smoked an average of 1.4±0.5 packs of cigarettes per day (28±10 cigarettes). The cumulative total of cigarettes smoked ranged from 20 to 100 pack-years. As shown in Table 1, levels of free F_2-isoprostanes in the circulation and F_2-isoprostanes esterified to plasma lipids were significantly higher in the smokers than in the nonsmokers (P=0.02 and P=0.05, respectively).

Table 1. Clinical and Laboratory Data on Subjects in the Pilot Study.*

Characteristic	Smokers (N=16)	Nonsmokers (N=8)	P Value
Sex (M/F)	15/1	8/0	—
Age (yr)	55.2±8.3	54.4±8.7	NS
Weight (kg)	79.1±11.8	83.0±12.7	NS
Height (cm)	177±7	177±8	NS
F_2-isoprostanes (pmol/liter)			
Free	166±58	90±52	0.02
Esterified	496±276	290±90	0.05

*Plus–minus values are means ±SD. NS denotes not significant.

Table 2. Clinical and Laboratory Data on Subjects in the Validation Study.*

Characteristic	Smokers (N=10)	Nonsmokers (N=10)	P Value
Sex (M/F)	5/5	5/5	—
Age (yr)	32.8±7.8	32.4±7.7	NS
Weight (kg)	74.1±15.9	69.1±14.2	NS
Height (cm)	173±11	170±8	NS
Cholesterol (mmol/liter)†	4.97±0.79	4.52±0.82	NS
Triglycerides (mmol/liter)‡	1.34±0.78	1.24±1.22	NS
F_2-isoprostanes (pmol/liter)			
Free	242±147	103±19	0.02
Esterified	574±217	345±65	0.03

*Plus–minus values are means ±SD. NS denotes not significant.
†To convert values to milligrams per deciliter, divide by 0.02586.
‡To convert values to milligrams per deciliter, divide by 0.01129.

Validation Study

The finding that levels of F_2-isoprostanes in the circulation were significantly higher among the smokers in the pilot study provided the impetus to conduct a more extensive, well-controlled study, not only to confirm the results of the pilot study but also to determine the short-term effects of smoking and the effects of abstinence from smoking on the production of F_2-isoprostanes. Unlike the pilot study, the validation study enrolled only young persons who had no apparent underlying disease and were taking no medications. In addition, equal numbers of male and female subjects were studied. The smokers in this study all smoked more than 1.5 packs of cigarettes per day (mean, 1.85±0.25 packs per day [37±5 cigarettes]). The smokers had from 4 to 50 pack-years of smoking (mean, 22±11). A variety of brands of cigarettes were used, but the mean tar content was 10.8±3.4 mg per cigarette and the mean nicotine content was 0.8±0.2 mg per cigarette. Additional characteristics of the subjects are summarized in Table 2.

Measurements of F_2-Isoprostanes

The levels of free F_2-isoprostanes in plasma from smokers (242±147 pmol per liter) were significantly higher than those measured in age- and sex-matched nonsmokers (103±19 pmol per liter, P=0.02) (Fig. 2). The levels of F_2-isoprostanes esterified to lipids in plasma from smokers (574±217 pmol per liter) were also significantly higher than those measured in nonsmokers (345±65 pmol per liter, P=0.03) (Fig. 2). No association was found between levels of F_2-isoprostanes in smokers and sex, age, weight, height, serum cholesterol or triglyceride levels, history of smoking in pack-years, number of cigarettes smoked per day, or the tar and nicotine content of the cigarettes smoked.

Measurement of the urinary excretion of prostanoid metabolites has proved to be an extremely reliable method of assessing the endogenous production of pros-

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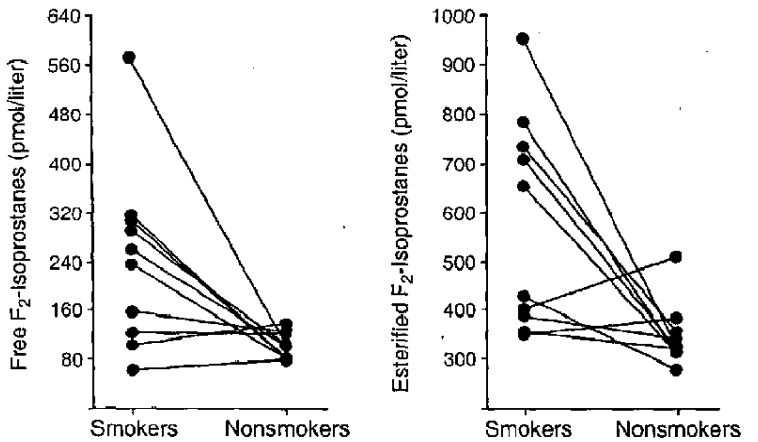

Figure 2. Levels of Free and Esterified F_2-Isoprostanes in Plasma from Smokers and Nonsmokers.

The dots representing subjects who smoked are each connected to a dot representing a nonsmoker matched to the subject for age and sex.

tanoids.[29] Therefore, we sought further confirmation of the overproduction of F_2-isoprostanes in smokers by quantifying the urinary excretion of F_2-isoprostane metabolites.[25] The urinary excretion of the metabolites in smokers (870±509 pmol per millimole of creatinine) was significantly higher than that in nonsmokers (415±155 pmol per millimole of creatinine, $P=0.05$). In addition, we found a significant correlation ($r=0.97$, $P<0.001$) between the urinary excretion of F_2-isoprostane metabolites and circulating concentrations of free F_2-isoprostanes in smokers and nonsmokers (Fig. 3).

We next determined the short-term effect of smoking on circulating levels of F_2-isoprostanes in the 10 smokers. We found no significant differences in the levels measured in blood obtained in the morning before smoking and the levels measured immediately after the subjects smoked three cigarettes during a 30-minute period. The mean plasma level of free F_2-isoprostanes was 242±147 pmol per liter before smoking and 237±117 pmol per liter after smoking ($P=0.43$). The mean level of F_2-isoprostanes esterified to plasma lipids was 574±217 pmol per liter before smoking and 624±214 pmol per liter after smoking ($P=0.20$).

Effect of Abstinence from Smoking on F_2-Isoprostane Levels

Eight of the 10 smokers who entered this study were able to stop smoking for two weeks. Levels of both free and esterified F_2-isoprostanes in the plasma of these eight subjects after two weeks of abstinence from smoking were significantly lower than the levels measured during smoking (Fig. 4). The mean free F_2-isoprostane level during smoking was 250±156 pmol per liter; after two weeks of abstinence it had fallen to 156±67 pmol per liter ($P=0.03$). The mean level of F_2-isoprostanes esterified to plasma lipids during smoking was 624±214 pmol per liter, and after two weeks of abstinence it had fallen to 469±108 pmol per liter ($P=0.02$). Cessation of smoking was confirmed by the finding that levels of urinary nicotine and cotinine measured during the smoking period (1.17±0.87 μmol per liter and 1.05±0.65 μmol per liter, respectively) fell to undetectable levels (<0.03 μmol per liter for each compound) in each subject after two weeks of abstinence from smoking.

Levels of Circulating Antioxidants

Plasma levels of uric acid, alpha-tocopherol, gamma-tocopherol, alpha carotene, beta carotene, lycopene, lutein and zeaxanthin, cryptoxanthin, total carotenoids, and retinol did not differ significantly between smokers and nonsmokers in the validation study ($P>0.05$). In accordance with the results of previous studies,[12-14] plasma ascorbate levels were significantly lower in the smokers (25.8±18.4 μmol per liter) than in the nonsmokers (54.3±19.1 μmol per liter, $P=0.002$); in this study we did not control for vitamin C intake.

Discussion

Our finding that the production of F_2-isoprostanes is higher in smokers than in nonsmokers provides compelling evidence that smoking causes oxidative modification of biologic components in humans. This conclusion is greatly strengthened by the finding that levels of F_2-isoprostanes in the smokers fell significantly after

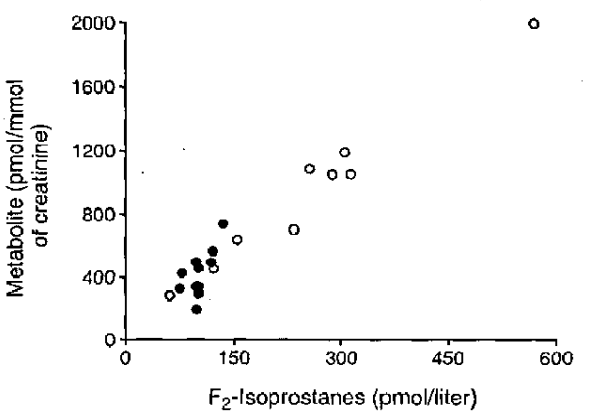

Figure 3. Levels of Free Circulating F_2-Isoprostanes and the Urinary Excretion of an F_2-Isoprostane Metabolite in Smokers (○) and Nonsmokers (●).

Each dot represents a different subject. The plasma levels of F_2-isoprostanes and the urinary level of F_2-isoprostane metabolite were highly correlated ($r=0.97$, $P<0.001$). There was a significant difference between smokers and nonsmokers with regard to the levels of both substances ($P\leq0.05$).

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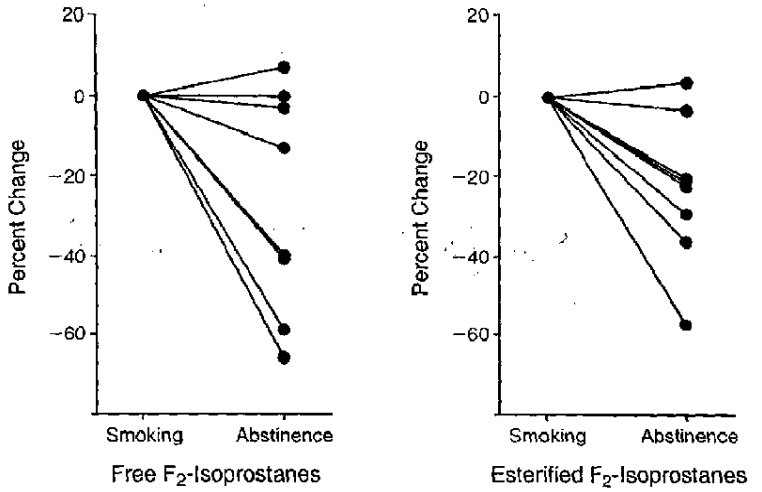

Figure 4. Percent Change in the Levels of Free and Esterified F_2-Isoprostanes in Plasma from Smokers after Two Weeks of Abstinence from Smoking.
Each dot on the right represents the change for an individual subject.

two weeks of abstinence from smoking. These results provide a basis for hypotheses that link oxidative damage of critical target biomolecules to the pathogenesis of diseases caused by smoking.

The results of previous studies of smoking and oxidative injury in humans have been conflicting and difficult to interpret. The most widely used test for oxidative stress is the measurement of malondialdehyde, a product of lipid peroxidation, by the thiobarbituric acid–reacting substances assay.[19] Harats and colleagues found no significant differences between smokers and nonsmokers in the levels of thiobarbituric acid–reacting substances in freshly prepared plasma or LDL.[30] However, other groups have reported that the levels of thiobarbituric acid–reacting substances are significantly higher in plasma from smokers than in plasma from nonsmokers.[16,30] The reason for these discrepancies may be that the measurement of thiobarbituric acid–reacting substances is not an accurate indicator of lipid peroxidation in biologic samples.[19] Another method of assessing lipid peroxidation in vivo is the measurement of exhaled alkanes, such as ethane and pentane; pentane exhalation has been reported to be higher in smokers.[31] However, the accuracy of exhaled pentane as a marker of endogenous lipid peroxidation has been questioned.[32]

On the other hand, we have shown previously that circulating levels of F_2-isoprostanes appear to provide an accurate measure of lipid peroxidation in vivo.[20,22,23] In the current study, we found higher levels of both free F_2-isoprostanes in the circulation and F_2-isoprostanes esterified to plasma lipids in smokers than in nonsmokers. The finding that levels of esterified F_2-isoprostanes were higher in smokers extends our previous findings from in vitro studies that F_2-isoprostanes are formed in plasma and LDL exposed to oxidative stress.[33] As previously discussed, oxidative modification of LDL is thought to be a key process in the development of atherosclerosis. Thus, the finding that plasma lipids from smokers have been modified by oxidation may provide a mechanistic link between cigarette smoking and atherogenesis. However, we did not show in this study that LDL itself was oxidatively modified. Although the vast majority of circulating lipids are associated with lipoproteins,[34] further studies will be required to determine to what extent LDL and other lipoproteins are oxidatively modified by cigarette smoking.

Interestingly, only slightly increased levels of F_2-isoprostanes, or none, were found in some of the smokers studied. This finding is of interest in view of the fact that some people appear more resistant than others to the toxic effects of smoking. The reason for this apparent heterogeneity in susceptibility to the oxidative effects of smoking is unclear. However, the ability to identify smokers who have only slightly increased levels of F_2-isoprostanes and those with more marked overproduction of F_2-isoprostanes may make it possible to explore the reasons for these differences. We did not find any relation between F_2-isoprostane production and age, sex, number of pack-years of smoking, or number of cigarettes smoked per day. It is possible that differences in antioxidant defense capacity affect susceptibility to the oxidant effects of cigarette smoke. In this regard, we did find, as have others, that smokers had substantially lower plasma concentrations of ascorbate than nonsmokers. Because we did not control for vitamin C intake, however, it remains to be determined whether this relation reflects increased utilization of ascorbate by smokers or decreased intake.

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